ORAL RECOMBINANT LACTOBACILLI VACCINES

Summary of the invention

The subject invention lies in the field of vaccine development. Specifically the invention is concerned with the development of oral vaccines. The invention involves the use of recombinant non pathogenic bacteria as carriers of an antigen capable of eliciting immune response after oral application. More specifically the invention covers the use of a lactic acid bacterium of the genus *Lactobacillus*. The lactic acid bacterium concerned is classified as a *Lactobacillus plantarum*. The antigen is expressed intracellularly and/or exposed on the surface of the Lactobacillus. The vaccine is a live vaccine in that it uses live microorganisms as carrier of the antigen. The invention also covers new recombinant *Lactobacillus plantarum* and expression vectors for use therein.

Background to the invention

For some time the skilled person has been entertaining the idea of development of an oral vaccine based on a non pathogenic carrier rather than using the currently commercially applied attenuated pathogenic microorganisms such as *Salmonella typhimurium* and *Mycobacterium bovis*. The development of such attenuated strains is tedious and lengthy. It requires development of recombinant strains that are no risk to the environment or individual due to elimination of the pathogenicity. They must however remain immunogenic for protective immune response to occur. Also a problem lies in the development of genetically stable recombinant strains. The use of an originally pathogenic microorganism carries the risk that immune reaction against the carrier itself will reduce the efficacy of a booster vaccine as will the presence of preexisting antibodies if the vaccinee has already been subjected to challenge by the carrier bacteria. Finally a large group of individuals will be more at risk using partially virulent carrier strains than using other carrier systems. This includes infants, the elderly and immuno-compromised individuals. In short there is a long felt need for vaccines that are safe and effective.

Mucosal vaccines i.e. vaccines using the mucosal delivery route appear to offer several advantages over systemic inoculation. They can be used to generate an IgA based immune response as a first line of defence. The protective immune response to mucosal infections is strongly dependent on the production of secretory IgA molecules. Such molecules are produced locally and are transported to the mucosal secretion products.

Another category of mucosal vaccines that could be useful aims at systemic immune response induction via mucosal application of the vaccine rather than parenteral application. Ideally the immune response should be equal to that of parenteral vaccines to be sufficiently effective.

The systemic application of antigens, however, may not work, or may not work reliably, in stimulating the mucosal immune system. Unfortunately neither does oral administration of specific antigens always result in sufficient immune response, if it even results in any response at all. This is caused by poor adsorption, rapid degradation in the gastrointestinal (G.I.) tract of the antigen and tolerance induction to the host. So in the past alternatives addressed.

Among the options attempted were the production of protective carrier systems like iscoms, microspheres and liposomes and the use of harmless live microorganisms for local production (i.e. *in situ* in the body) of desired antigens in vivo.

The group of lactic acid bacteria has seemed a suitable starting point when looking for a group of harmless microorganisms to be used as antigen carriers. They are used on a large scale use in the food production technology. They form a group of food grade (i.e. usually with GRAS-status) gram positive microorganisms suitable for human consumption. They have a long record of safe use in food products. An additional interesting aspect in their favour is of course the absence of LPS (which appear in some pathogenic microorganisms currently used as carriers). Thus as no problems with endotoxic shock risks would be anticipated they are apparently safe to apply via the mucosal route.

In addition, lactic acid bacteria are produced as probiotics due to the colonising, i.e. capable of settling and or growing, of cavities such as mouth, urogenital or gastrointestinal tracts, where they play a role in maintaining a balanced

normal microflora. To date however no oral vaccine using non pathogenic bacteria is commercially available.

In the field of mucosal vaccines one must distinguish nasal (and vaginal) vaccines on the one hand and oral vaccines on the other. The environment that oral antigens are faced with is eminently more hostile than the nasal environment. In addition it has been demonstrated that vaccines capable of eliciting immune reactions on nasal application have not been successful upon oral application. Nasal vaccines using non pathogenic carriers have been suggested, but due to the non-predictive character or efficacy these are of no assistance when faced with the problems of developing an oral vaccine. For instance, the use of recombinant *Lactobacillus casei* and *Lactobacillus plantarum* strains as or in nasal vaccines have been described and both are equally suitable for this route of administration. Surprisingly, in the present invention, it has been found that *Lactobacillus plantarum* strains are suitable for use as or in an oral vaccine. In contrast - as further discussed below - the art has not been able to obtain comparable results with other Lactobacillus strains, such as *L .casei*, that have been suggested as nasal vaccines (Pouwels et al., J. Biotech. 1996 44:183-192; Pouwels et al., Int. J. Food Microbiol. 1998 41:155-167).

There are 3 categories of lactic acid bacteria based vaccines that have been suggested in the prior art as potentially being suitable. For two of these categories oral vaccines appear to be potentially feasible. Firstly there are the *Lactococcus lactis* based vaccines and secondly the *Streptococcus gordonii* based vaccines. Both categories have various advantages and disadvantages which are well documented (Mercenier A. in Probiotics: A critical review, Horizon Scientific Press, Wymondham, U.K. 1999, pp 113-127; Pouwels et al., Int. J. Food Microbiol. 1998 41:155-167).

The third category is that of the *Lactobacillus* based vaccines and includes nasally introduced recombinant Lactobacilli capable of inducing immune response. However as yet there is no orally introduced recombinant Lactobacilli that has actually induced an immune response. Instead there are speculative disclosures of recombinant non pathogenic bacteria expressing heterologous antigens *in vivo* for producing a significant immune response upon oral application of the bacterium.

One study discloses an unsuccessful experiment involving oral introduction into mice of a recombinant *Lactobacillus* expressing a heterologous antigen intracellularly (Wells et al., Antonie van Leeuwenhoek 1996 70:317-330). *Lactobacillus plantarum* 80, expressing *E. coli* β-galactosidase intracellularly, was introduced orally into mice on days 0,1 and 2. Subsequent oral boosting occurred after a 4 week interval. This experiment revealed no significant antibody responses to the β- galactosidase (the heterologous antigen), a protein not known for any immunogenic properties. This result was in contrast to intraperitoneal results where an immune reaction was achieved, using the same bacterial strain, the same antigen and the same expression system. Numerous explanations for this failure can be put forward, but none of so far have clarified the lack of good results for oral vaccination.

Other art refers to the potential use of recombinant *Lactobacillus plantarum*. WO99/11284 suggests that an oral vaccine can be produced using lactic acid bacteria. The embodiment suggested seems to be one where soluble protein is secreted from the recombinant microorganism. No mention of surface exposition or intracellular production is provided and a large number of lactic acid bacteria are speculatively put forward as being useful. The suggested bacteria are *Lactobacillus delbruekii*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus coryniformis*, *Lactobacillus brevis*, *Lactobacillus leichmannii* and strains of *Lactobacillus* isolated from intestinal flora such as *Lactobacillus rhamnosus* 901. The latter is indicated as being especially preferred as it possesses resistance both to acid and bile juices. The lactic acid bacterium mentioned in the Examples is *Lactobacillus plantarum* 8826 (3×10^9 bacteria/ml, twice a day for 18 days).

The lactic acid bacteria according to WO99/11284 should produce urease as heterologous antigen. No other antigen is suggested and illustration of successful induction of an immune response *in vivo* is provided. The example supposedly illustrating this is merely speculative in nature and no actual results are provided. Also it suggests that the antigen (urease) was excreted by the bacterial host into its surroundings as a "free", soluble protein (i.e. not exposed on or otherwise associated with the surface of the bacterial host).

There are in fact serious doubts as to whether the postulated method would be successful in providing an immune reaction. Numerous articles have been published pointing out that the secretion of soluble protein is unlikely to induce an immune response: therefore, this is not likely to be a suitable technique for providing a useful oral vaccine. This in combination with the absence of any experimental data, would seem to illustrate a lack of enablement of an oral vaccine using a *Lactobacillus* as host. At best it is a speculative disclosure of use of a *Lactobacillus* secreting soluble protein urease as heterologous antigen with a view to treating *Heliobacter pylori* associated disease. Nothing is disclosed concerning other antigens that might be expected to have specific problems e.g. the unpredictability of their surviving the aggressive environments of oral/gastrointestinal media.

The review article (Mercenier of 1999) describes numerous constructs expressing heterologous antigens (e.g. TTFC) that have been introduced into *Lactobacillus plantarum* NCIMB 8826 (a human saliva isolate). For all these recombinant Lactobacilli constructs nasal introduction in mice resulted in an immune response. In addition it indicates that oral tests have also been carried out for comparison but no results for the oral experiments are presented and there is no mention of where in the cell the antigens are expressed. Thus there is also no mention of whether any, some or all of the constructs produced any significant immune reaction. The absence of data in combination with general knowledge concerning the extrapolation of data regarding nasal vaccines to oral vaccines, together with previous failure using Lactobacilli as oral vaccines would appear to suggest lack of success. Certainly the general interpretation would be that there was not a reasonable expectation of success.

A similar situation applies to an abstract from the Mercenier group (Institut Pasteur, Lille: Grangette *et al*, Immunology letters, 69(1): No. 1, page 176: 45.13) where no data is given to support the claims of immunisation.

This conclusion is supported by Pouwels *et al* 1998 where it is reported how expression vectors in *Lactobacillus casei* achieved good expression of heterologous antigen but only provided fairly low levels of antibodies after oral introduction. This could be due to the poor viability of *L. casei* but this parameter has not been further examined and there are numerous other reasons for the failure of the recombinant

Lactobacillus casei to provide protective immune response. For example, the amount of expression *in vivo* may have been too low to actually elicit immune response or the antigen was not presented in a manner enabling it to induce an immune response.

In the experiments leading to the present invention recombinant *Lactobacillus casei* was included as a comparison with the *L. plantarum* vaccine of the invention. This confirmed the lack of efficacy of a *Lactobacillus casei* based expression system as an *in vivo* carrier of heterologous antigens. This finding questions the disclosure of WO99/11284 as there it was suggested *Lactobacillus casei* could be an example of a useful host.

To further support how extrapolation of data in this field between species is not always reliable, previous experiments have compared recombinant *L. plantarum* and non recombinant *L. casei* for eliciting adjuvant activity. *L. casei* was shown to be the best microorganism eliciting *in vivo* adjuvant activity. Thus one could speculate that *L. casei* would be the preferred candidate for a vaccine over *L. plantarum* or any other strain of lactic acid bacteria.

Description of the Invention

A first aspect of the invention is an (e.g. oral) vaccine comprising a recombinant lactic acid bacterium expressing a heterologous antigen (suitably *in vivo*). The antigen can be expressed intracellularly and/or (exposed) on the surface of the lactic acid bacterium. This can thus be a specific immunogenicity eliciting component for eliciting immunogenicity against the heterologous antigen, or can elicit an (immune) response. The recombinant lactic acid bacterium is preferably a *Lactobacillus* organism, such as *L. plantarum*, and optionally at least one pharmaceutically acceptable carrier suitable for use in a formulation for oral delivery is present.

In the present description, the terms and phrases mentioned below have the following meaning, unless indicated otherwise.

"*Bacterial host*" means the bacterium or bacterial strain that is used to express the desired antigen(e.g. via recombinant techniques). This is administered to a human or animal (mammal) in or as part of a vaccine in order to illicit an immune

response against the antigen. The term is used to designate the native strain transformed to express the antigen, the recombinant strain expressing the antigen (also referred to separately as "*recombinant host strain*"), or both.

"*Mucosal delivery (route)*" of a vaccine means any route of administration to the body of a human or animal for which it is not required to penetrate or puncture the skin (e.g. as with intravenous, intramuscular, subcutaneous or intraperitoneal administration). Usually, this means that the vaccine is administered to the body via one of the body cavities, such that it comes into contact with the mucosa. Hence mucosal administration in particular refers to nasal, oral and/or vaginal administration.

"*Mucosal vaccine*" means any vaccine suited, adapted, intended and/or formulated for mucosal delivery.

"*Oral delivery (route)*" (of a vaccine) means any route of delivery to the body of a human or animal into, by which the vaccine can be presented to, the gastrointestinal (G.I.) tract or any part thereof. Usually, this will involve administration into or via the mouth into the G.I. tract. "*Oral administration*" also includes administration directly into the G.I. tract or into any part thereof, and including into the stomach, for instance using a tube or catheter.

"*Oral vaccine*" means any vaccine suited, adapted, intended and/or formulated for oral delivery as defined above.

A response (e.g. an antibody response or immune response) is deemed "*significant*" if it leads to a detectable change or response in a human or animal, and in particular to a detectable immunological change or response, such as the production of antibodies, cytokines, lymphokines, etc. Tests for determining whether a response is "*significant*" are known in the art and include, but are not limited to, titration of antibody levels in biological samples using ELISA techniques, ELISPOT techniques and *in vitro* lymphocyte stimulation assays. Such techniques are usually carried out on a biological sample, such as a biological fluid or cell sample, obtained from the human or animal.

A "*significant*" response may be, but is not necessarily, also a "*protective*" response as defined below. A response (e.g. an immunological response against a pathogen or an antigen) is deemed "*protective*" when it is capable of protecting the

human or animal having the response against the pathogen and/or against a pathogen associated with the antigen.

An antigen is deemed "*exposed*" on a bacterial host (also be referred to as "*(surface) exposition*" of the antigen) when it is present, forms part of, is attached to, and/or is otherwise associated with or detectable on (e.g. using a suitable immunological detection technique such as FACS or immunofluorescent microscopy) the surface of the bacterial host (e.g. the bacterial cell wall or envelope)

Preferably, "*exposed*" means that the bacterium - when presented to a cell of a human or animal that is capable of mediating an immune response (such as the cells of the G.I. tract mentioned below) for a sufficient time and in a sufficient amount - is capable of eliciting a "*sufficient*" immune response against the antigen.

Bacterial Strains

A *Lactobacillus plantarum* strain can readily be determined using known parameters (e.g. Bergeys Manual of determinative bacteriology and Vescovo *et al*, Ann. Microbiol. Enzymol. 43 261-284 (1993)). The skilled person can therefore readily determine whether a lactic acid bacterium is a *Lactobacillus plantarum*. Numerous *Lactobacillus plantarum* strains have been deposited at various institutes and are readily available.

The native strain selected as the bacterial host should preferably have GRAS (generally regarded as safe) status, and more preferably be food grade.

Also, the bacterial host used should allow - upon transformation with an appropriate construct encoding an antigen- expression of the desired antigen either intracellularly and/or exposed on the surface.

Preferably the level of expression of the antigen - e.g. intracellulary and/or exposed on the surface as determined by SDS-polyacrylamide gel electrophoresis or FACS should be at least from 1-5 % of the total cell protein, or alternatively 80% or more, preferably 100% or more, of the level of expression provided by *L. plantarum* strain 256 under the same conditions and using the same expression vector.

Also, the bacterial host is preferably capable of settling in and/or colonizing at least part of the gastrointestinal tract, such as the mouth, the throat, the larynx, the

gut, the small intestine, the large intestine, the ileum and/or the colon, or a combination thereof. Preferably the bacterial host is such that it mainly settles in the intestine, more preferably in the small intestine or caecum.

The (recombinant) *Lactobacillus plantarum* strain preferably exhibits a persistance in the individual to be immunized (upon oral administration and as determined by the presence of the strain in the faeces) of at least 5 days, preferably at least 9 days, and suitably more than 15 or even 20 days. Longer persistance may not be required if an administration regimen comprising the use of one or more booster immunisations as described below.

A preferred embodiment exhibits a persistance longer than that of *L. plantarum* 80 and preferably longer than that of *L. plantarum* NCIMB 8826 under equivalent conditions. This can be assessed objectively in the gastrointestinal tract of mice, preferably of the same age and under equivalent conditions of treatment although options available.

Preferably the *L. plantarum* strain is not LMG 9211 (NCIMB 8826 as described by Mercinier above), DSM 4229 *L. casei* 393, and/or *L. plantarum* 80. Preferably the bacterial host is one of the following *L. plantarum* strains: 256, LMG 1284, LMG 6907, LMG 8155, LMG 9205, LMG 9206, LMG 9208, LMG 9209, LMG 9210, LMG 9212, LMG 11405, LMG 11460, LMG 8095, LMG 8027, LMG 12167, LMG 13556, LMG 17552, LMG 18021, LMG 18023, LMG 18024, LMG 18027, LMG 18095; 386, 299 , 105 or 275 (see Molin et al., 1993. J. Appl. Bacteriol. 74:314), 299v (see WO 96/29083); So5,36^{E}, 95, 120 or 44 (see Johannson et al 1995, Int. J. Syst. Bacteriol, Vol 45(4):670-675), 79, 107, 98, 53, 97, 101 or 125 (see Johansson et al 1995 Int. J. Food. Micro. 25:159), CH, ATCC 8041, ATCC 10012, ATCC 10776, WCFS, DF66 IIIa, DF66spez. -IVa, and/or the *L. plantarum* strains available from the Japanese Collection of Micro-organisms under the accession numbers: 8341, 8342, 8343, 8344, 8345, 8346, 8347 and/or 8348.

Preferably the *Lactobacillus* bacterium employed is foreign to the individual (human or animal) to which it is to be administered, for example vaccinated. The bacteria of the invention are modified or recombinant, and it is so the wild-type or naturally occurring bacteria, that is to say the non-recombinant or unmodified *Lactobacillus*, that is suitably foreign. Thus by foreign it is intended to refer to a

Lactobacillus strain that the individual (or humans) has not encountered before. In other words, it is preferred to use a *Lactobacillus* strain that is non-human, for example it is not found or present in humans. Preferably the strain will be one not found in humans or animals, for example a strain that does not exist in the gut (G.I. tract). Such strains will provoke a greater or larger immune response. Thus although some prior workers have used human-derived *L. plantarum* strains (such as NCIMB 8826, a human saliva isolate) the present inventors took a counter-intuitive approach and looked for strains not present in humans.

For example, prior workers have used the *L. plantarum* strain deposited as NCIMB 8826. This strain is found in humans, for example it exists already in the gastrointestinal tract. This means that it is unlikely to provoke or elicit as good an immune response as a *Lactobacillus* strain that is foreign to (or not present in) humans.

The present invention therefore preferably uses a *Lactobacillus* strain that is not found in the mucosa (non-mucosal) or G.I. tract of individual, for example it is not endogenous (to humans or a species of animal to be vaccinated). Indeed, the most preferred strains (e.g. *L. plantarvm* 256) are found in silage. Such strains are clearly foreign to humans, and so provide an increased immune response. Nevertheless, preferably the strain may be capable of adhering to the intestinal mucosa.

The strain may also be of non-foodstuff origin, for example not found in (human) foods. This may thus exclude some *L. casei* strains, e.g. 393. Strain 393 is found in cheese, and so preferably strains found in dairy or fermentation products are excluded. This means that the individual has not (or may not) have had contact (e.g. ingested) the strain, and so using strains not found in foods is more likely to provoke an immune response. This may explain the long persistence times found with certain *Lactobacilli*. It may be that those bacteria are cleared more slowly as they are not recognised by the immune system. Suitably the strain is thus of animal origin, for example from an animal feedstuff (e.g. silage). Strains that are generally more suitable are commensal (in the gut), rather than dietary (e.g. dairy origin).

Preferably the *Lactobacillus* strain is viable (or alive) and intact. Suitably

they will be able to persist (in the mucosa) of the individual for at least 7 days. This can easily be tested using procedures known in the art (for example, testing for the existence of the organism in faeces).

Thus the invention additionally relates to a non-human and/or non-human food *Lactobacillus* bacterium, such as *L. plantarum*, which has been modified to express a heterologous antigen (intracellularly and/or on the cell surface). This bacterium is preferably able to elicit an immune response in an individual, to whom the bacterium is administered. Thus the naturally occurring or unmodified *L. plantarum* is preferably foreign to that individual, for example it is not endogenous to humans or the animal to which it is to be administered. Preferably the chosen *L. plantarum* strain will not be present in the G.I. tract or mucosa of humans or that species of animal.

The invention also relates to an *L. plantarum* bacterium which has been modified to express an heterologous antigen intracellularly and/or on the cell surface, to elicit immune response to an individual and which can persist in the gastrointestinal tract of that individual for at least 7 days.

Other suitable strains may be selected by the skilled person on the basis of one or more of the following properties or factors:

- stability of the construct encoding the antigen in the bacterial host selected; level of expression of antigen in the bacterial host selected; regulation of expression of antigen in the bacterial host selected; site of expression of antigen in the bacterial host selected; and/or stability of antigen produced;
- the biochemical properties of the strain used, for example its sugar fermentation profile (API), cell wall composition, structure of LTA, structure of peptidoglycan, 16S RNA sequence, acid resistance, bile acid resistance, agglutination properties, adjuvanticity, immune modulating properties, *in vitro* adherence properties, mannose-specific adherence, presence of proteinaceous adherence factors, presence of mapA-like adherence factors and/or presence of large proteinaceous adherence factors with repeated amino acid sequences; and/or
- the interaction of the bacterial host with cells of the individual to which the host to be administered (i.e. as part of a vaccine according to the invention) including

but not limited to its persistence , viability, *in vivo* expression of antigen and/or tissue-specific persistence.

In at least some, preferably most, and more preferably essentially all of these properties, the strain used should be essentially (at least) equivalent to the strains mentioned above, and more preferably equivalent to *L. plantarum* 256, e.g. as determined on the basis of tests/assays for these properties known *per se* in the art. After a suitable host has been selected, it may be transformed with a genetic construct as described herein, after which its suitability as an oral vaccine may be tested, i.e. using the tests and protocols described in the Examples later. It is envisaged that on the basis of the description herein, and optionally after carrying out - for the purposes of confirmation- the tests described herein, the skilled person will be able to identify other *L. plantarum* strains suitable for use as or in vaccines of the invention.

Preferably the *L. plantarum* is in the group (or cluster) 1, (which includes strains 101, 97, 53, 256, ATCC 14917, 36^E, 95, 98, 299, 299v, 107, 105, 79, 275, 386, So5 and ATCC 8014), suitably subgroup (or subcluster) 1b (which includes 256, ATCC 14917, 36^E, 95 and 98). Particularly preferred is the *L. plantarum* strain 256.

A combination of one or more of the strains mentioned above may also be used.

Recombinant aspects

A preferred embodiment of the invention is a vaccine wherein the recombinant *Lactobacillus plantarum* comprises an expression vector capable of expressing the heterologous antigen intracellularly and/or such that the heterologous antigen is exposed on the cell surface under conditions present in the gastrointestinal tract.

Any embodiments of recombinant *Lactobacillus plantarum* in the form of a vaccine wherein the heterologous antigen is specific for inducing immunogenicity against a pathogenic microorganism are covered by the scope of the invention. The host may express a heterologous antigen specific for mucosa colonising pathogens or pathogens entering the body via the mucosa, specifically via the oral route. The

heterologous antigen can be specific for a gastrointestinal tract colonising pathogen. A heterologous antigen specific for tetanus (*Clostridium tetanus*), such as TTFC, is a particularly suitable candidate.

The recombinant bacterial hosts (e.g. of *Lactobacillus plantarum)* can comprise expression vectors capable of expressing the heterologous antigen intracellularly and/or such that the heterologous antigen is exposed on the cell surface to a degree sufficient to induce protective immunogenicity.

Preferably the vaccines are formulated such that a single dose is sufficient. However embodiments where multiple applications over a period of time, e.g. with a view to the persistence of the bacterial host in the G.I. tract, are also envisaged. The provision of booster vaccinations is also envisaged with the vaccine formulations according to the invention.

A preferred administration regimen comprises one or more "initial" doses or administrations on any of days 1 to 4, followed by one or more booster administrations on any of days 14 to 21, and optionally one or more further booster administrations on any of days 28 to 25. A single initial administration, followed by a single booster administration, within this time period, will generally be sufficient.

Surprisingly, in some cases, it has been found that, for example when a bacterial host is used with a persistance between 6 to 12 days, a significant immune response was obtained essentially only after the first booster administration. This was despite the fact that at the time of the first booster - i.e. days 14 to 21- the bacterial host (i.e. from the initial administration) was not longer detectable in the faeces of the individual. The later Examples show that identically primed mice were all boosted at the time the oral vaccine was re-administered, either 2,3 or 4 weeks after initial priming. In one aspect, the invention therefore relates to a method and preparations suited for such an administration and boosting regimen.

The invention also relates to a vaccine comprising a recombinant *Lactobacillus plantarum* (suitably comprising expression vector(s)) capable of expressing the heterologous antigen intracellularly and/or such that the heterologous antigen is exposed on the cell surface to a degree that exceeds either that of the vector disclosed for *Lactobacillus plantarum* 80 β-galactosidase expression or of *Lactobacillus plantarum* 80 expressing a galactosidase.

As high a degree of expression as possible without damaging the viability of the cell or the host to be vaccinated is envisaged. With higher expression, less frequent and lower doses may be required for immunisation purposes. Naturally the dosage regime will not only depend on amount of antigen but also on antigen type and the presence or absence of other immunogenicity stimulating factors in the vaccine.

A high degree of expression can be achieved by using homologous expression and/or secretion signals on the expression vectors present in the recombinant *Lactobacillus plantarum* in the vaccine. Suitably expression regulating signals as present in the constructs in the Examples are useful. Other expression signals will be apparent. The expression vector can optimise expression depending on the *Lactobacillus strain* it is incorporated in.

Surprisingly, it was found that expression vectors that gave reasonable levels of expression in *Lactobacillus casei* (but not to a degree sufficient for the recombinant *Lactobacillus casei* to be contemplated as an oral vaccine), provided sufficient expression for *Lactobacillus plantarum* to provide immunogenicity upon oral administration. Thus *Lactobacillus plantarum* comprising expression vectors capable of expression in *Lactobacillus casei* are covered. This was not expected to be the case, firstly because of the lack of sufficient expression in *Lactobacillus casei* for vaccine use and secondly due to the unpredictability of expression levels between various lactic acid bacteria.

The preferred *Lactobacillus plantarum* is *Lactobacillus plantarum* 256 as this strain provided good results, better than *Lactobacillus casei* under equivalent conditions.

Antigens

The antigen is able to elicit or stimulate an immune response, and so can be any antigen against which an immune response, more specifically a "significant" immune response and/or a "protective" immune response as defined above, can be elicited in an animal (preferably a mammal such as a human). The antigen will usually be associated with a pathogen, disease state and/or disorder of the human or animal to which the vaccine is to be administered. Preferably the antigen will be able to interact with one or more (e.g. specific) receptors, for example present on lymphocytes or in antibodies released from them. The antigen can thus be an immunogen. Since the antigen will generally be one that can elicit an immune response, this will usually exclude enzymes (e.g. urease, β-galactosidase), for example a protein that is present already in the individual. Antigens are thus preferably foreign to that individual.

Any antigen, antigenic component or epitope known *per se* that can be expressed in the microbial host can be used. Usually this will be a peptide, a protein, or an antigenic part or fragment thereof, such as an epitope. As such, it may either be a native antigenic peptide or protein (or part, fragment or epitope thereof) or an antigenic analog or mutant thereof, for instance obtained synthetically or using recombinant DNA techniques.

Recombinant bacteria and/or bacterial strains, as well as vaccines based thereon, may be provided that can be used to illicit a significant immune response, and preferably a protective immune response, against various antigens. Suitable antigens include:

- allergen;
- viral and/or bacterial antigens including those from (e.g. the gp160 envelope protein of) the HIV virus, a surface glycoprotein (of a *Leishmania* parasite), Shiga-like toxin, *Shigella* lipopolysaccharide antigen, *Escherichia coli* fimbrial antigen, a CFA antigen (of an enterotoxigenic *Escherichia coli* strain), anthrax toxin, pertussis toxin, tetanus toxin;

– antigens from such pathogens as herpes virus, rubella virus, influenza virus, mumps virus, measles virus, poliomyelitis virus, rotavirus, respiratory syncytial virus, *Campylobacter* species, *Chlamydial* organisms, species of the genus *Cryptosporidium*, cytomegalovirus, human immunodeficiency virus, *Actinomyces* species, *Entamoeba histolytica*, arenaviruses, arboviruses, *Clostridium botulinum*, species of the genus *Candida*, *Vibrio cholera*, *Cryptococcus neoformans*, EHEC strains of *E.coli* O157:H7, O26:H11, O111:H8 and O104:H21, ETEC strains of *E. coli*, strains of *E.coli* shown to possess enteroinvasiveness (EIEC), EPEC strains of *E.coli*, EAggEC strains of *E.coli.*, DAEC strains of *E.coli*, filoviridae, parvovirus, *Filarioidea*, *Staphylococcus aureus*, species of the genus *Clostridium perfringens*, *Helicobacter pylori*, Caliciviruses, *Giacardia lamblia*, *Neisseria gonorrhoeae*, hantaviruses, hepatitis viruses types A, B, C, D, E, *Legionellae* strains, *Mycobacterium leprae*, *Listeria monocytogenes*, species of the genus *Clostridium perfringens*, *Borrelia burgdorferi*, *Pseudomonas pseudomallei*, Epstein Barr virus, *Onchocerca volvulus*, Poxviruses, *Bordetella pertussis*, *Yersinia pestis*, *Coxiella burnetti*, rabies virus, *Treponema pallidium*, *Mycobacterium tuberculosis*, *Salmonella typhi*, eukaryotic parasite causing malaria, *pneumocystis pneumonia*, as well as agents causing toxoplasmosis.

Preferably the allerben is a human allergen, or an allergen provoking an allergic reaction in the type or species of individual to whom the composition is to be administered. It may be a house or insect allergen, such as from dust mite, e.g. Der p 1.

Apart from native antigens and antigenic components (including antigenic parts, fragments or epitopes thereof), antigenic mutants or analogues thereof - obtained synthetically or via recombinant DNA techniques - may be used. Also, in the vaccines of the invention, a combination of two or more such antigens may be present or expressed. These antigens may be expressed by a single (type or strain) of bacterial host, or by several different (types or strains) of bacterial host.

Of the above, antigens against and/or specific for rotavirus, respiratory syncytial virus, *Mycobacterium tuberculosis*, human immunodeficiency virus, *E.coli*,

Vibrio cholera, streptococci and chlamydia are especially preferred for use as an antigen in the vaccines of the invention.

The antigen is preferably such that, upon expression, it still allows – at least to some extent, which may be reduced compared to the native strain - the recombinant bacterial host to settle in and/or colonize (part of) the gastrointestinal tract upon administration, and to persist there. This may be for a time sufficient to provide a significant immune response against the antigen and/or the pathogen associated with it.

The antigen may be expressed in the bacterial strain using any expression system known *per se* that expresses the antigen in the recombinant bacterial host in a manner that makes the host suitable for use in the vaccine. This means that at least the antigen should be expressed intracellularly and/or such that it becomes exposed on the surface of the bacterial host, and at a suitable level, e.g. at least 1 - 5 % of total cell protein(as measured by SDS-polyacrylamide gel electrophoresis and standard protein staining).

Expression systems

Usually, the expression system will comprise a genetic construct comprising at least one nucleotide sequence encoding the desired antigen(ic component), preferably operably linked to a promoter capable of directing expression of the sequence in the bacterial host. Suitably the antigen to be expressed can be encoded by a nucleic acid sequence that is adapted to the preferred codon usage of the bacterial host . The construct may further contain (all) other suitable element(s) , including enhancers, transcription initiation sequences, signal sequences, reporter genes, transcription termination sequences, etc., operable in the selected bacterial host.

The construct is preferably in a form suitable for transformation of the bacterial host and/or in a form that can be stabley maintained in the bacterial host, such as a vector or plasmid. More preferably, a food grade construct is used.

A particularly preferred construct according to the invention comprises the multi-copy expression vector described in PCT/NL95/00135 (WO-A-96/32487), in

which the nucleotide sequence encoding the antigen has been incorporated. Such a construct is particularly suitable for expression of a desired protein or polypeptide in a lactic acid bacterium, in particular in a *Lactobacillus*, at a high level of expression, and also can be used advantageously to direct the expressed product to the surface of the bacterial cell. The constructs (e.g. of PCT/NL95/00135) may be characterised in that the nucleic acid sequence encoding the antigen is preceded by a 5' non-translated nucleic acid sequence comprising at least the minimal sequence required for ribosome recognition and RNA stabilisation. This can be followed by a translation initiation codon which may be (immediately) followed by a fragment of at least 5 codons of the 5' terminal part of the translated nucleic acid sequence of a gene of a lactic acid bacterium or a structural or functional equivalent of the fragment. The fragment may also be controlled by the promoter. The contents of PCT/NL95/00135, including the differing embodiments disclosed therein, and all other documents mentioned in this specification, are incorporated herein by reference.

Preferably the construct used provides a level of expression - e.g. intracellularly and/or exposed at the surface - that is at least comparable to the level provided by a vector of PCT/NL95/00135 in the same bacterial host under the same conditions.

The vaccines of the invention are preferably oral vaccines, that is to say they are adapted for oral administration. Such oral vaccine compositions will usually be alkaline, since usually alkali is required in order to neutralise acid in the stomach, and allow the bacteria (or at least most of them) to pass through the stomach into the intestine alive. It is preferred that most of the bacteria administered will survive the stomach, and pass into the intestine. Increased immune responses can be achievable when the bacteria are alive, that is to say viable, rather than dead. This is because they can continue to express the heterologous antigen *in vivo*. Not all pharmaceutical formulations will be alkaline, and therefore those that are not alkaline (for example nasal formulations) will not be suitable for oral administration.

The sequence encoding the antigen can be obtained from any natural source and/or can be prepared synthetically using well known DNA synthesis techniques. The sequence encoding the antigen can then (for instance) be incorporated in a suitable expression vector to provide a genetic construct of the invention, which is

then used to transform the intended bacterial host strain (for instance as described in PCT/NL95/00135).

The recombinant bacterial host thus obtained can then be cultured, upon which the harvested cells can be used to formulate the vaccine, optionally after further purification and/or processing steps, such as freeze-drying to form a powder.

The techniques required to create the genetic constructs containing the antigen-encoding sequence and for transforming, culturing and harvesting the bacterial hosts are well known in the art. For instance they are described in PCT/NL95/00135 as well as in standard handbooks, including Sambrook et al, "Molecular Cloning: A Laboratory Manual" (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory (1989) and F.Ausubel *et al*, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York (1987).

The vaccine comprising the bacterial host can be formulated in a known manner, such as for the formulation of vaccines and/or for the formulation of preparations of live bacteria for oral administration to an animal or human. Reference can be made to preparations for the administration of probiotics, e.g. for the treatment of gastrointestinal disorders.

The vaccine according to the invention can be in a form suitable for oral administration, which may be a solid, semi-solid or liquid form, including but not limited to solutions and/or suspensions of the bacteria, which are usually preferred.

The vaccine preparation may also be in the form of a powder, such as a freeze dried powder that can be reconstituted before use, e.g. using a suitable liquid. It may be in the form of a solid or liquid preparation that is (to be) mixed with solid, semi-solid or liquid food prior to administration. It may also be in the form of a fermented product.

Besides the bacteria, the vaccine may contain one or more pharmaceutically acceptable carriers or excipients, such as water. The vaccine may also contain one or more adjuvants, including immune adjuvants, suitable for oral administration. These are compatible with the bacterial host and suitably do not interfere (too much) with its desired immunogenic properties. According to one embodiment, the adjuvants may be a lactic acid bacterium, such as the bacterial host strain itself, one of the other *L. plantarum* strains mentioned above, another *Lactobacillus* species, or even a

Lactococcus, Bifidobacterium or *Propionibacterium* species suitable for oral administration to humans or animals.

Also, the vaccine may contain one or more further therapeutic substances and/or one or more substances that can facilitate and/or enhance the colonization of (part of) the G.I. tract by the bacteria, and/or the growth of the bacteria in the G.I. tract. The preparation may also be in a form suitable for (direct) administration into the stomach or gut, for instance via a tube or catheter. Preferably upon oral administration the bacterial host settles in, and may thereupon colonise, the gastrointestinal tract, or at least a part thereof, such as the mouth, the gut, the small intestine (e.g. duodenum, jejunum or ileum), the large intestine (or part thereof, such as the caecum) or colon, and preferably either the small intestine or the caecum.

Immunogenicity

The antigens expressed by the bacterial host thus can come into contact with the mucosal layer, the lining and/or the wall of the G.I. tract (collectively referred to hereinbelow as "*wall of the G.I. tract*"), and more specifically with cells within this wall. This can mediate an immune response against the antigen(s) thus presented, such as antigen-presenting cells (for example macrophages, dendritic cells and/or B-lymphocytes). This immunological response by the cells within the wall of the G.I. tract may by itself already constitute a significant immune response as defined herein, and/or it may trigger further immunological reactions/responses in the body of the human or animal to which the vaccine has been administered, which again may be a significant response and/or may be a protective response as defined herein. However, the invention is not limited to any specific mechanism via which the recombinant bacterial host elicits any immune response(s). For instance, the immune response elicited by an antigen as expressed by the bacterial host may provide a stronger or enhanced immune response compared to the response that would be elicited by the antigen as such, e.g. as a (free) soluble protein.

In the invention the manner in which the antigens are presented and/or delivered to the wall of the G.I. tract and/or to specific cells therein - such as the cells which mediate and/or are involved in the immune response - may be improved or

enhanced when compared to the administration, expression and/or use of the antigen as a free soluble protein. For example, as the bacterial host can adhere to the wall of the G.I. tract and can persist *in situ* for a long(er) period of time, it may be that the antigens are presented locally at the wall of the G.I. tract in a large(r) amount, at a high(er) level or concentration, in a more stable or resistant manner and/or for a long(er) period of time. Any of these may thus providing an (enhanced) immune response.

The bacterial host - or any part or fragment thereof and/or any further compound(s) produced by it - may interact with the wall of the G.I. tract and/or with specific cells therein such as the cells which mediate and/or are involved in the immune response. This may enable, facilitate or enhance the immune response to the antigens associated with the bacterial host, such as compared to administration, expression and/or use of the antigen as a free, soluble protein.

Although the antigen is preferably expressed by the bacterial host such that the antigen becomes exposed on the cell surface, it is not excluded that - in order to illicit the immune response - the contents of the cells of the bacterial hosts are *in situ* (i.e. locally at the wall of the G.I. tract) released and/or liberated from the bacterial cell, e.g. by a mechanism which makes the walls of the bacterial cell wall permeable and/or destroys the cells or the bacterial cell wall. Thus it is possible that (*in situ* at the wall of the G.I. tract) the immunogenic response may not be (just) caused/elicited by the intact bacterial host, but by a part, fragment, fraction or compound thereof for example the antigen as such and/or cell fragments or cell fractions comprising the antigen. Thus although it is preferred that the vaccines comprises intact, viable and/or live bacteria, vaccines that (e.g. also) contain fragments, fractions, lysates etc., of or derived from the recombinant bacterial host are not excluded.

Dosages

The amount of bacteria administered is not critical, but suitably it is sufficient for the bacteria to settle into and/or colonize (the desired part of) the G.I. tract, and/or to cause a significant immune response. A suitable amount will be at least 10^8 cfu, preferably 10^8 - 10^{10} cfu per dose. This may allow a sufficient amount of bacteria to

pass into the intestine, if required. Oral administration of doses less than 10^8 cfu may not always give the desired immunogenicity (at least not in a reliable manner), whereas amounts of more than $5x10^{10}$ cfu, if cumbersome to administer orally, are less preferred. The above amounts of bacteria (dosages) may correspond, to for instance, 10^6 to 10^8 cfu per kg of body weight of the human or animal. The concentration of bacteria in the vaccine (or other formulation) may be at least $5x10^9$/ml, such as at least 10^{10}/ml. The formulation may be administered for only up to 2, 3 or 4 days. The bacteria may still be detectable in the individual at least 5 days, 7 days or 9 days after the first or last administration.

Vaccines

Preferably the individual to be vaccinated is a human or an animal. The human can be an infant, immunocompromised person, elderly person or a normally healthy infant, child or adult.

One advantage of the bacterial hosts used is that they can be capable of surviving in/passing through the gut in amounts sufficient to colonize the intestine(s). Nevertheless, one can administer the bacteria in or as a coated or encapsulated preparation, for instance in the form of a delayed release composition or an enteric coated composition. Suitable encapsulating compounds include but are not limited to chitosan, maltodextrin, lipids and oligo- and polysaccharides. Encapsulation may also improve the shelf-life of the vaccine. The vaccine may be adjuvant-free but preferably contains one or more adjuvants.

Although the invention has been mainly described with reference to *L. plantarum* strains there may possibly be strains of other *Lactobacillus* species which may also prove suitable for use as a bacterial host, for example in vaccines of the invention. These can include strains from *L. pentosus*, *L. reuteri*, *L. animalis* (= *L. murinus*), *L. fermentum*, *L. acidophilus*, *L. crispatus*, *L. gasseri*, *L. johnsonii*, *L. salivarius*, *L. brevis*, *L. rhamnosis* and/or *L. paracasei*.

The strains useful in the invention preferably have GRAS status and more preferably are food-grade. Also, they are most preferably used in combination with the expression vectors of PCT/NL95/00135 mentioned above, or another vector that

gives a level of expression (e.g. intracellularly and/or exposed on the surface of the bacterial host) comparable to this preferred expression vector.

Furthermore, although the use of strains belonging to the genus *Lactobacillus* are preferred, it is envisaged that suitable strains could possibly also be selected from bifidobacteria and the propriobacteria, e.g. from the genus *Bifidobacterium* and/or the genus *Propriobacterium.* Suitable strains can be selected by the skilled person in the same way as *L.*plantarum and/or Lactobacillus can be selected.

For instance, a suitable test for determining/confirming whether a selected strain is suitable as a bacterial host according to the invention, is to transform the host with the TTFC carrying vector pLP401 (for surface anchored/surface exposed expression of the TTFC-antigen) and/or the TTFC carrying vector pLP503 (for intracellular expression of the TTFC-antigen), then to administer the recombinant host thus obtained orally to an animal, preferably a mammal (e.g. a mouse such as a BALB/c and/or C57bl/6 mouse), preferably according to the single dose priming and boosting schedule as mentioned in the Examples, followed by measuring the end-point titres of IgG in individual sera by ELISA using tetanus toxoid. In such an assay, the selected recombinant host preferably provides higher (i.e. at least 1% higher) titres than *L.plantarum* NICMB 8826 and/or *L.plantarum* 80 when transformed with the same vector and administered under the same conditions; and more preferably titres which are at least 10% higher, even more preferably at least 20% higher.

Preferably the selected recombinant host preferably provides titres which are at least 70%, more preferably at least 90% of the titres provided by *L.plantarum* 256 transformed with the same vector and administered under the same conditions, and even more preferably titres which are at least equal to the titres provided by *L.plantarum* 256 transformed with the same vector and administered under the same conditions.

The invention additionally relates to a bacterium which has been previously described for use in the vaccines of the invention. The invention additionally relates to expression vectors suitable for intracellular expression, or exposure on the cell surface, for heterologous antigen. This expression will be in a bacterium, such as a *Lactobacillus plantarum* as previously described.

The invention additionally relates to the use of a Lactobacillus (e.g. *L. plantarum*) bacterium which has been modified to express a heterologous antigen (e.g. intracellularly and/or on the cell surface) for the manufacture of vaccine for an individual to whom the unmodified *L. plantarum* is foreign. The unmodified *L. plantarum* is preferably not found in humans (or in human foods), or for example is not present in the G.I. tract or mucosa of a mammal. The bacteria as described herein can be used in the manufacture of vaccine. This vaccine may be adapted for oral administration. Preferably the bacterium will elicit any immune response on administration. The vaccine of the invention can particularly be used for or preventing tetanus.

Where the law allows, the invention also relates to the administration of the bacterium or vaccine to an individual, such as a human or animal (e.g. a mammal), where that individual (or subject where appropriate) is in need of bacterium or vaccine. The individual may require treatment or prophylaxis or a particular disease, as described above.

Preferred features and characteristics of one aspect of the invention are equally applicable to another aspect *mutatis mutandis*.

The invention will now be illustrated by means of the following Examples, which refer to the figures, which are provided merely for the purposes of illustration, and the invention is not to be construed as being limited to the subsequent Examples.

EXAMPLES

General Issues

The delivery of antigens to mucosal-associated lymphoid tissues in paediatric and immuno-compromised populations by safe non-invasive vectors, such as commensal lactobacilli, represents a crucial improvement to prevailing vaccination options. The oral and nasal immunisation of mice is described with vaccines constructed for heterologous gene expression in *Lactobacillus* in which the 50kDa fragment C of tetanus toxin (TTFC) is expressed either as an intracellular or surface-exposed protein. The data indicate that the strain *Lactobacillus plantarum* is more

effective than *Lactobacillus casei*. Immunisation of mice with live recombinant lactobacilli induced significant levels of circulating TTFC-specific IgG following nasal or oral delivery of vaccine strains. In addition, sIgA in bronchoalveolar lavage fluids, as well as antigen-specific antibody-secreting cells and antigen-specific T-cell activation in draining lymph nodes following nasal delivery were induced, substantiating their potential for safe mucosal delivery of paediatric vaccines.

Live microbial vaccine-vectors viable at target sites of mucosal immunisation represent efficient delivery systems to facilitate immune responses at mucosal and systemic sites concurrently. Observations to date have underlined the superiority of attenuated pathogenic viruses and bacteria over non-replicating antigens for the induction of mucosal immune responses. Oral subunit vaccine-approaches based upon peptides or purified recombinant proteins may therefore be deficient in this one important requisite, the induction of protective immunity in the G.I.-tract itself. Oral vaccination remains safe and inexpensive whilst maintaining the potential for single-dose immunity, contributing therefore to improved compliance rates in vaccination programs. Currently, vaccination against tetanus involves tetanus toxoid (TT) formulations and maintains poor coverage and contamination concerns that run concurrent with all needle-delivery vaccines. Tetanus toxin fragment C (TTFC) is the 50kDa non-toxic papain cleavage product of the tetanus holotoxin and is an alternative protective immunogen and is currently utilised in several live-vector systems under development. The delivery of vaccine subunits to the mucosal surfaces by a suitable live microbial vector is a rational response to the obstacles encountered by parenteral vaccines. However, potential safety and environmental considerations, particularly the immune status of the vaccine recipients in developing countries, still negates the employment of the majority of mucosally delivered vector-candidates such as *Escherichia coli, Salmonella* and Vaccinia virus. Therefore, non-pathogenic, food grade or commensal bacterial vectors have begun to receive attention for their vaccine potential.

Commensal bacteria maintain a sophisticated non-invasive ecology with the host and although surveyed by the immune system are not necessarily susceptible to immune clearance from their ecological niches. The predominance of lactobacilli in various regions of the aero-digestive tracts indicates their particular potential as live oral

vaccines. Their "generally recognised as safe" (GRAS) status is evident from applications in the food industry and their capacity to enhance immune responses has been demonstrated with co-administered DxRRV rhesus-human reassortent oral rotavirus vaccine and TNP-conjugated antigen, effects probably attributable to the macrophage-activating and IFN-γ inducing properties of the Gram-positive peptidoglycan and lipoteichoic acid fractions. Immune homeostasis at the mucosa, in which lactobacilli participate, is a combination of physical exclusion, IgA secretion and active regulation by T-cell subsets Possibilities were investigated to avoid antigen-specific peripheral tolerance following oral delivery (oral tolerance) by vaccinating with TTFC in particulate form (contrasting soluble dietary antigens), to permit processing and presentation by mechanisms ordinarily contributing to immune regulation of intestinal flora. The data indicate that the strain *Lactobacillus plantarum* 256 is more effective as compared to *Lactobacillus casei* 393 and that delivery of TTFC expressed as an intracellular antigen was, in some situations, slightly better than cell-surface expression (under the conditions tested and the detection techniques employed).

Recombinant DNA techniques.

E. coli DH5α was used as a host strain for manipulation of the previously described pLP401 or –503 shuttle vectors(Maassen *et al*, Vaccine 17: 2117, 1999). The 1329 bp DNA coding for TTFC (A. Mercenier, Lille, France) was elongated at its 3' end with *Xho*I and *Nco*I restriction sites using PCR techniques, to facilitate cloning into the pLP401 and pLP503 shuttle vectors.

Prior to transfer of the plasmids into lactobacilli by electroporation, the T*ldh* terminator sequence present in the shuttle vectors was removed by *Not*I digestion resulting in the pLP401 or pLP503 plasmids shown in Table 1 below.

Table 1: Bacterial strains and plasmids used

Strain or plasmid	**Relevant genotype or phenotype**	**Source/ reference**
L. plantarum	256, wildtype	This study
L. casei	393	ATCC
pLP503-TTFC	P-*ldh*, Amp^r, Ery^r, TTFC	Pouwels *et al.*, 1996, supra
pLP401-TTFC	P α-*amy*, Amp^r, Ery^r, ssAmy, Anchor, TTFC	Pouwels *et al.*, 1996, supra

Key: p-*ldh* = promoter sequence of *ldh* gene of *L. casei*, p α -amy = promoter sequence of α -amylase gene of *L. amylovorus*, Ery^r = erthromycin resistance gene, Amp^r = ampicillin resistance gene, ssAmy = sequences encoding secretion signal (36 aa) of α -amylase gene of *L. casei*, Anchor = anchor peptide (117aa) encoding sequences of *L. casei*, TTFC = 1329 bp DNA coding for the fragment C of tetanus toxin.

Re-ligation of the vectors juxtaposed the TTFC protein encoding sequences in frame with the codons of the translation initiation region present downstream of the regulatable amylase gene (α-amylase) or constitutive lactate dehydrogenase (*Ldh*) gene promoter sequences present in pLP401 and pLP503 respectively. Following electroporation of competent lactobacilli, transformants were selected on erythromycin (5μg/ml) agar plates. General molecular cloning techniques and transformation of lactobacilli were carried out using known techniques (Maassen *et al*, supra).

***Lactobacillus* strain selection**

Lactobacillus spp. appropriate as host strains for transformation were identified by quantitative cultures of faecal samples obtained following inoculation of mice with single intra-gastric doses of 10^9 cells of a wide panel of rifampicin-resistant wild-type lactobacilli. *L. plantarum* 256 (P. Conway, Sydney, Australia or Adlerberth *et al*, Appl

& Env. Microbiology, Vol. 62(7): 2244-2251, 1996) which persisted in the G.I. tract for up to 12 days and *L. casei* (ATCC 393) which became undetectable within 72 hours, were selected as prototype host-strains.

Gel electrophoresis and Western blotting.

Recombinant *L. casei* and *L. plantarum* containing the plasmids detailed in Table 1 above were routinely prepared from glycerol stocks by semi-anaerobic overnight (o/n) growth of a 1:50 dilution in MRS medium containing 5μg/ml erythromycin.

Transformants with plasmids containing the constitutive *ldh* promoter were optimally grown in antibiotic selective LCM medium with 2% (w/v) glucose at 37°C. Transformants with plasmids containing the regulatable α-amy promoter were grown by diluting an o/n culture of cells 1:50 in mannitol (2% w/v) containing LCM medium. Total cell extracts were obtained from the bacteria by sonicating cells four times on a 30 sec on/30 sec off cycle using a W870 Branson™ sonicator, to release both cytoplasmic or cell membrane-bound proteins.

Proteins in 30μg of total cell extracts or fractions were separated by SDS-polyacrylamide gel electrophoresis (PAGE), (10% acrylamide, 400mM Tris [pH 8.9]) and run in a 25mM Tris, 192mM glycine buffer (pH 8.3) at 200V for 45 mins. Protein was transferred electrophoretically onto nitrocellulose using a Biorad™ electrophoresis unit. Immunoblots were developed using optimally diluted rabbit TTFC-specific antiserum and goat-anti-rabbit IgG-specific phosphatase conjugates (Nordic, Tilburg, The Netherlands).

Flow-cytometric analysis of cell-surface expression of TTFC./

At defined time points bacteria were prepared for analysis by FACScan. Cells were washed twice and re-suspended in PBS/1% Bovine serum albumin (BSA). 50μl of optimally diluted Rabbit TTFC-specific antiserum was added to the cells for 1 hour. Cells were again washed twice and bound antibody was detected by a 30 min incubation with fluorescein isothiocyanate-conjugated (FITC) anti-rabbit at a dilution of 1:1000. Cells were then washed twice prior to analysis for light scatter and fluorescence on a Becton-Dickinson flow cytometer. A gate was set around appropriate size events

as determined by cytograms of forward and side scatter. Controls were prepared by staining wild-type *L. casei* 393 or *L. plantarum* 256, staining recombinants using non-immune rabbit serum or excluding the rabbit TTFC-specific antiserum. All procedures were performed on ice with 1% BSA. For each sample data was collected for 10,000 - 20,000 gated events. The fluorescence obtained from bacterial cell suspensions was represented by fluorescence histogram and mean channel intensities calculated.

Immunisation.

BALB/c or C57BL/6 mice, age 6-8 weeks, were immunised intra-gastrically (o) or intra-nasally (i.n.) with preparations of recombinant *L. casei* or *L. plantarum* expressing TTFC. Bacteria obtained from the o/n cultures were diluted 1:50 in MRS or LCM medium containing 1% glucose and grown for 6 hours at 37°C until an OD695nm of between 0.6 - 0.8 (mid-exponential phase) was reached. Cells were pelleted by centrifugation at 4°C, washed once with PBS and appropriate concentrations of bacteria prepared in sterile PBS.

For oral immunisation 2-5x10^9 cells were administered intra-gastrically in a 250µl volume of 0.2M $NaHCO_3$ on three consecutive days. For intra-nasal immunisation 2-5x10^9 cells were administered to the nares of non-anaesthetised mice in 20µl volume of PBS. Control mice received identical doses of wild type lactobacilli. Plate counts were performed with all inoculum samples to confirm CFU amounts administered to the mice.

Sample collection and ELISA.

Serum was prepared from blood samples obtained from the tail vein from pre-immune mice and subsequently at 7 day intervals beginning 21 days following immunisation.

To obtain brocho-alveolar lavage samples, mice were sacrificed at specific time-points and the lungs were canulated and inflated repeatedly with 0.7 ml of PBS/0.1%BSA, following which the collected wash volume was centrifuged at 1000g and the supernatants stored at –80°C.

Antigen-specific immunoglobulin G (IgG) levels were evaluated using microtiter plates coated o/n with 50µl of a 0.16µg/ml solution of TT (RIVM, Bilthoven,

The Netherlands). Individual serum samples were titrated by serial log_2 dilutions and assayed in duplicate. Bound antibody was detected by the addition of 50µl of optimally diluted goat anti-mouse IgG-phosphatase conjugate (Nordic, Tilburg, The Netherlands). Following the addition of the PNPP (1mg/ml in 0.1M DEA/ $MgCl_2$) chromogen substrate, antibody levels were quantified by measuring plate A405nm values obtained 30-90 minutes following the initiation of reaction. End-point titres were calculated using a cut-off determined from the mean absorbance (OD 0.2) of a 1:10 dilution of serum obtained from pre-immune mice. For antigen-specific immunoglobulin A (IgA) levels in broncho-alveolar lavage fluid the same procedure was performed, using an optimally diluted goat anti-mouse IgA phosphatase conjugate (Nordic).

Antigen-specific T lymphocyte proliferation assays and ELISPOT.

At 12 and 21 days following the last immunisation, the spleens and cervical lymph nodes (CLN) of mice were removed aseptically. Single cell suspensions were prepared by passage through a cell strainer (70uM Nylon; B&D,), and centrifugation at 1500rpm for 10 min. Viable, un-fractionated cell numbers were assessed by Trypan blue dye exclusion.

For testing antigen-specific T-lymphocyte proliferation, cells were resuspended and plated at concentrations of $3x10^5$ cells/spleen or $5x10^5$ cells/LN in a final volume of 200µl culture medium (RPMI-1640, supplemented with 10% heat-inactivated fetal calf serum, 2 mM-L-glutamine, 20U/ml penicillin and 20 µg/ml streptomycine (all Gibco, Pairsley, UK), and 50µM 2-mercaptoethanol {Sigma, MO}) in sterile flat-bottomed 96-well culture plates (Nunc, Denmark). Control wells contained medium only, and antigens were added to triplicate cultures over the indicated dose range. All cells were maintained in a humidified 5% CO_2 atmosphere at 37°C for 4 days. The cells were pulse-labelled with 0.6uCi[^{3}H] thymidine (^{3}H-TdR, 5 Ci/mMol, TRA 120, Amersham, UK) in 30 µl volumes/culture well during the last 16-18 hours before harvesting. Cells were collected using an ILACON cell harvester and deposited onto glass fibre filter discs (Whatman). Thymidine incorporation was assessed by gas scintillation spectrometry (ß-plate counter, Canberra Packard, Meriden, CT) and the results were calculated as the mean c.p.m (± SD) from triplicate cultures and expressed as a stimulation index (SI).

Quantifying the number of TT-specific antibody-secreting cells (ASC) in the spleen and CLN was undertaken according to Czerkinsky *et al*,J. Immunol. Methods 65: 109 (1983). Microtiter plates (Maxisorp plates, Nunc, Denmark) were coated o/n with 50μl of a 0.16μg/ml solution of TT (RIVM, Bilthoven, The Netherlands) or 50μl of PBS as control. After extensive washing, and blocking of the plate with PBS/0.1%BSA, cells were added at concentrations of $1x10^6$ and $2x10^5$ cells/spleen or $5x10^5$ and $1x10^5$ cells/LN into a final volume of 50μl culture medium and incubated for 4h in a humidified 5% CO_2 atmosphere at 37°C. The plates were rinsed and incubated for 20 min. with ice-cold PBS containing 10mM EDTA to remove the cells, and washed again with PBS/0.05% Tween-20, and PBS/0.5% BSA. Bound antibody was detected by the addition of 50μl of optimally diluted rabbit anti-mouse Ig phosphatase-conjugate (DAKO, Denmark) o/n at 4 °C. Plates were washed extensively, and incubated with 1mg/ml BCIP in AMP buffer containing 1% low melting temperature agarose. The plates were inverted over a light source, and macroscopically blue dots were scored.

Expression of recombinant TTFC in lactobacilli.

Expression of cytoplasmic or cell wall bound TTFC by *L. plantarum* and *L. casei* transformants was demonstrated by collection of cells in mid-exponential phase followed by sonication to disrupt the bacteria. Proteins were separated by SDS-PAGE and immunoblots developed using TTFC-specific rabbit antiserum.

Specifically, pLP401-TTFC transformants (surface-anchored expression) were grown in LCM (+2% mannitol) and pLP503-TTFC transformants (intracellular expression) in MRS (Difco), (both supplemented with 5μg/ml erythromycin), at 37°C to an OD 0.6, pelleted and disrupted by sonification. 30μg total protein was analysed on a 10% SDS/polyacrylamide gel and separated proteins transferred to nitrocellulose electrophoretically. TTFC was visualised with rabbit anti-TTFC (1:500) and a phosphatase/PNPP chromogen combination. Bars indicate the migration of molecular weight markers.

Immuno-fluorescence analysis was performed of recombinant *L. plantarum* pLP401-TTFC and *L. casei* pLP401-TTFC expressing TTFC as a surface-anchored product. Lactobacilli were gated on the basis of forward and side scatter and stained

with rabbit TTFC-specific antiserum diluted 1:500 (Calbiochem, Ca). Bound antibody was detected with optimally diluted FITC-conjugated anti-rabbit IgG (Jackson, PA). Fluorescence levels from cells collected at OD 0.6 were analysed by FACScan (Becton Dickinson) and shown in histogram form, presented in relation to levels of fluorescence obtained with non-recombinant lactobacilli. 10,000 cells were analysed in each experiment.

From the photograph of the protein expression and the Mackintosh generated FACscan it was shown that lactobacilli containing pLP503-TTFC express only the intracellular 50kDa TTFC polypeptide. *L. plantarum* containing the vector pLP401-TTFC express a surface-anchored 75kDa polypeptide corresponding to the 50kDa TTFC fused to an anchor sequence of 25kDa, at a level higher than for *L. casei* pLP401-TTFC. Exposition of TTFC on the cell-wall of *L. plantarum* and *L. casei* through fusion to the anchor sequence was confirmed by FACscan.

TTFC-specific antibody responses after intranasal immunisation with recombinant lactobacilli.

Serum was collected from pre-immune mice and at 7 day intervals beginning on day 7. TTFC-specific serum IgG in individual or pooled serum was measured by ELISA using microtitre plates coated o/n at 4°C with 0.16μg/ml of tetanus toxoid in PBS. Bound antibody was detected by the addition of anti-mouse AP conjugate and PNPP substrate. OD_{405nm} values of each well were measured at 90 mins. End-point titres were determined using a cut-off value calculated as the mean OD+ 2 SD's (≈0.2) of pre-immune sera diluted 1:10. Table 2 shows the results of the following three protocols.

A. 3 BALB/c mice were immunised intra-nasally with 3 doses of $5x10^9$ *L. plantarum* pLP503-TTFC or 3 doses of $5x10^9$ *L. casei* pLP503-TTFC in 20μl of PBS on days 1-3. Identical booster immunisations were administered on days 28-30.

B. 16 C57BL/6 mice were immunised with 3 doses of $5x10^9$ *L. plantarum* pLP503-TTFC intra-nasally in 20μl of PBS or orally in 200μl of $NaHC0_3$ on days 1-3. Identical booster immunisations were administered on days 28-30

C. 3 C57BL/6 mice were immunised with $5x10^9$ *L. plantarum* pLP503-TTFC intra-nasally in 20µl of PBS on day 1 and day 28 or 3 doses of *L. plantarum* pLP401-TTFC intra-nasally in 20µl of PBS on days 1-3 followed by a booster with either $5x10^9$ *L. plantarum* pLP503-TTFC on days 28-30, or *L. plantarum* pLP401-TTFC on days 28-30 and 49-51, intra-nasally in 20µl of PBS.

BALB/c and C57BL/6 mice receiving three i.n. doses of *L. plantarum* or *L. casei* expressing intracellular TTFC on days 1-3 were strongly primed for a secondary response to TTFC following booster i.n. administrations on days 28-30. The titre of the TTFC-specific response in BALB/c mice following immunisation with recombinant *L. plantarum* was higher than recombinant *L. casei* (Table 2A), with IgG detectable as rapidly as day 28, rising to titres of $10^{3.5}$ and $10^{2.9}$ by day 49, respectively.

Table 2A: Titre (Log_{10}) vs days post immunisation

	Days	0	23	35	42	49
intranasal *L.plant* TTFc	mean*	0.0	0.0	1.2	3.5	3.4
	St. Dev	0.0	0.0	0.43	0.63	0.75
intranasal *L.casei* TTFc	mean	0.0	0.0	0.3	2.95	2.8
	St. Dev	0.0	0.0	0.60	0.78	0.50

* end-point titre (TT-specific IgG)

C57BL/6 mice demonstrated mean end-point titres higher (though not significantly) than BALB/c mice (Table 2B) and therefore were selected for further analysis by i.n. immunisation using a single-dose priming and booster schedule with the *L. plantarum* transformants that expressed TTFC intracellularly (Table 2C).

Table 2B: Titre (log_{10}) vs days post immunisation

	Days	0	23	35	42	50
oral *L.plant* TTFc	mean*	0.000	0.100	0.881	1.350	0.986
	St. Dev	0.000	0.400	1.234	1.319	1.258
intranasal *L.plant* TTFc	mean*	0.000	1.840	4.275	4.219	4.163
	St. Dev	0.000	1.172	0.338	0.417	0.407

*end-point titre (TT-specific IgG)

Table 2C: Titre (log_{10}) vs days post immunisation

L.plantarum pLP401-TTFC / booster with *L.plantarum* pLP401-TTFC

DAYS	0	14	28	42	49	56	63	70	77	88
mean*	0	0	0.2	0.1	0.05	1.55	1.9	2.2	2.7	2.8

L.plantarum pLP401-TTFC/booster with *L.plantarum* pLP503-TTFC

DAYS	0	14	28	42	49	56
mean*	0	0	0	2.7	3.4	3.95

L.plantarum pLP503-TTFC/booster with *L. plantarum* pLP503-TTFC

DAYS	0	14	28	42	49
Mean*	0	0	1.2	4.0	4.1

* end-point titre (TT-specific IgG)

In comparison to the results in Table 2A obtained in BALB/c mice, higher mean titres of serum IgG ($10^{4.2}$) were induced in C57BL/6 by day 49. However, regardless of whether a triple- or single-dose priming and boosting immunisation schedule was used,

C57BL/6 mice demonstrated serum IgG endpoint titres booster that were not significantly different.

In contrast, in order to induce TTFC-specific serum IgG in C57BL/6 mice immunised i.n. with *L. plantarum* expressing TTFC at the cell surface, it was necessary to prime mice on days 1-3 and subsequently boost at least twice (on days 28-30 and 49-51) before antigen-specific responses were measurable (Table 2C). Interestingly however, mice primed as above, with the *L. plantarum* pLP401-TTFC did induce TTFC-specific serum IgG (with more rapid kinetics than responses obtained from naive mice), when boosted on days 28-30 using the *L. plantarum* pLP503-TTFC transformant instead (Table 2C). This observation, intriguingly, implies that antigen-specific sensitisation of the mice has in fact occurred following administration of *L. plantarum* transformed with pLP401-TTFC, although maturation or measurability of the immune response is for numerous reasons prevented from occurring. In contrast however, *L. casei* expressing TTFC on the cell surface failed to demonstrate any priming effect in either BALB/c or C57BL/6 strains as did the *L. plantarum* transformant itself administered on days, 1, 28 and 56 as single doses.

Induction of tetanus-toxoid specific IgA antibodies was investigated in bronchoalveolar lavage fluids after intra-nasal immunisation of mice with *L. plantarum*-pLP503-TTFC. C57BL/6 mice were immunised at days 1-3 with $5x10^9$ *L. plantarum* pLP503-TTFC, followed by an identical booster immunisation at days 28-30, either i.n. (A & B) or orally (C & D). At 12 (A & C) and 21 (B & D) days, after the last boost 4 animals per group were sacrificed and bronchoalveolar lavages were obtained by flushing the lung through a canule with 0.7ml PBS/0.1%BSA. TT-specific IgA in these samples was measured by ELISA using microtitre plates coated o/n at 4°C with 0.16μg/ml of tetanus toxoid in PBS. Bound antibody was detected by the addition of anti-mouse AP conjugate and PNPP substrate. OD_{405nm} values of each well were measured after o/n at 4°C.

As shown in Tables 3A & 3B C57BL/6 mice receiving three doses of *L. plantarum* expressing TTFC on days 1-3 were primed mucosally for a TT- specific IgA response in broncho-alveolar lavage fluids measured on 12 and 21 days following booster i.n. administration on days 28-30. Bronchoalveolar lavages obtained from mice

immunised i.n. with wild-type *L. plantarum* 256 demonstrated no reactivity with TT coated onto microtitre plates at either day 12 or day 21 respectively.

Tables 3A, B, C and D: OD_{405nm} vs days after intranasal (A,B) or oral (C,D) boost

12 days (A) in. *L. plant*	*1	2	3
mouse 1	**0.503	0.42	0.353
mouse 2	0.545	0.501	0.439
mouse 3	0.356	0.297	0.263
mouse 4	0.246	0.193	0.174

21 days (B) in *L. plant*	1	2	3
mouse 1	0.435	0.368	0.306
mouse 2	0.34	0.268	0.256
mouse 3	1.059	0.78	0.645
mouse 4	0.648	0.508	0.43

12 days (C) oral *L. plant*	1	2	3
mouse 1	0.233	0.22	0.21
mouse 2	0.211	0.198	0.206
mouse 3	0.219	0.209	0.191
mouse 4	0.205	0.188	0.179

21 days (D) oral *L. plant*	1	2	3
mouse 1	0.167	0.177	0.138
mouse 2	0.196	0.167	0.169
mouse 3	0.187	0.181	0.165
mouse 4	0.2	0.164	0.145

*dilution factor

** OD_{405nm} (TT-specific IgA ELISA)

TTFC-specific antibody responses after oral immunisation with recombinant *lactobacilli*

In a comparative study of intra-nasal versus oral immunisation, C57BL/6 mice received three doses of *L. plantarum* expressing intracellular TTFC on days 1-3, followed by booster administrations on days 28-30. Following oral immunisation, induction of TTFC-specific serum IgG responses occurred in 9 out of the 16 mice tested (Table 2B). After i.n. immunisation all 16 mice responded with high TTFC-specific endpoint titres, with a mean titre of $10^{4.3}$ within 7 days of the booster, remaining high for 3 weeks. The mean endpoint titres of the oral responders was $10^{1.6}$ at day 7, and reaching a peak of $10^{2.4}$ at 14 days post boost. In contrast to the intra-nasal group, in orally immunised mice no TT-specific IgA response could be measured in bronchoalveolar lavages at either day 12 or day 21 respectively (Table 3 C & D).

In an additional study, mice were orally primed on days 1, 2 and 3 and boosted either 2, 3 or 4 weeks later. Irrespective of the timing of the boost, a TTFC-specific IgG response was observed within 7 days of the booster inoculation.

In contrast, BALB/c or C57BL/6 mice immunised orally with the *L. casei* transformants (expressing TTFC either intracellularly or surface-anchored), or *L. plantarum* expressing TTFC on the cell surface failed to induce detectable TTFC-specific serum IgG responses at all equivalent time points examined. Mice receiving wild-type lactobacilli or irrelevant vectors demonstrated no TTFC-specific serum IgG responses at any time point (data not shown).

TT-specific antibody-secreting cells and T cell responses in spleens and CLN

C57BL/6 mice were immunised either orally or intranasally on days 1-3 with either $5x10^9$ *L. plantarum* pLP503-TTFC transformants or the wild-type *L. plantarum* 256 as control. This was followed by an identical booster immunisation on days 28-30. At 12 days or 21 days following the last boost, mice were sacrificed and spleens and CLN cell suspensions were prepared.

Table 4: Enumeration of tetanus –toxoid specific antibody-secreting cells per 10^6 cells in spleens and cervical lymph nodes 12 and 21 days after the last boost.

	12 days		21 days	
groups*	spleens	CLN	spleens	CLN
oral L. plant. 256	0#	0	0	0
oral L. plant. TTFC	0	0	0.4 (0-2)	0
i.n. L. plant 256	0	0	0	0
i.n. L. plant. TTFC	48 (7-107)	195 (156-233)	16 (4-31)	13 (5-27)

Key: *16 C57Bl/6 mice per group were immunised at days 1-3 intra-nasally (i.n.) or orally with $5x10^9$ *L. plantarum* (either *L. plantarum* 256 or *L plantarum* pLP503-TTFC transformants), followed by an identical booster immunisation

at days 28-30. 12 Days or 21 days after the last boost per group 8 animals were sacrified and spleens and cervical lymph nodes (CLN; pooled per 2 animals) cell suspension were prepared. The amount of TT-specific Ig producing cells were determined by ELISPOT.

#The data, presented as antibody-secreting cells (ASC) per 10^6 cells, represent the mean (range) of 8 spleens samples or 4 CLN samples, measured in triplicate wells.

In Table 4 the number of TT-specific antibody secreting cells (ASC), determined by ELISPOT, present in spleen or CLN are shown. Following i.n. immunisation with *L plantarum* transformants, high numbers of TT-specific ASC are found in spleens and CLN of i.n. immunised mice, at 12 days after the last boost, and these numbers have decreased by day 21. These data suggest that antigen-specific sensitisation of the mice has occurred locally at the CLN level, as well as systemically in the spleen. In the orally immunised group 2 out of 8 spleen cell suspensions tested contained TT-specific ASC, whereas no ASC can be found in the CLN.

Induction of antigen-specific T-cells by intra-nasal immunisation of mice was investigated with *L. plantarum*-pLP503-TTFC. C57BL/6 mice were immunised at days 1-3 intra-nasally (i.n.) or orally with $5x10^9$ *L. plantarum* (either *L. plantarum* 256 or *L plantarum* pLP503-TTFC transformants), followed by an identical booster immunisation at days 28-30. 12 Days (A & C) or 21 days (B & D) after the last boost per group 8 animals were sacrificed and spleens (A & B) and CLN (pooled per 2 animals)(C & D) cell suspension were prepared. The cells were examined for [^{3}H] thymidine incorporation following *in vitro* incubation of $3x10^5$ spleen cells or $5x10^5$ CLN cells per well for 72 hours with TTFC, TT, TT peptide P30 or medium alone. [^{3}H] thymidine was added to the cultures in the last 18 hours. Results are expressed as the SI calculated from the mean cpm of triplicate test cultures of cells divided by the mean cpm of cultures receiving buffer alone. The background values of cultures receiving buffer alone varied from: A 220-760 cpm, B: 150-240 cpm, C 1000-4000 cpm, and D 150-560 cpm.

In Figure 1 the antigen-specific T-cell responses of spleen or CLN are presented. In mice immunised i.n. with the *L. plantarum* pLP503-TTFC

transformants, proliferation is measurable following re-stimulation with either TTFC, TT or TT peptide P30, suggesting that i.n. immunisation induced specific immunity initially via local lymph node activation. Antigen-specific T cell responses of spleen and CLN were also obtained in BALB/c mice. No antigen-specific proliferation was observed in cells obtained from mice immunised orally or identical routes with wild-type *L. plantarum* (Fig.1).

The pLP401- /503- plasmids enabled directed (-surface or intracellular) and regulatable expression of TTFC at levels and efficiencies not previously attainable in *Lactobacillus*. In addition, the *L. plantarum* pLP503-TTFC that expressed TTFC intracellularly was demonstrated to be very immunogenic following intranasal delivery, priming on days 1-3, and boosting at days 28-30. Immunogenicity was shown at the systemic level by high TT-specific IgG serum titres, as well as at the mucosal level, showing TT-specific IgA in the BAL fluids. In addition, TT-specific ASC and antigen-specific T-cell proliferation were demonstrated at the systemic level in spleens as well as locally in cervical LN. Following oral delivery of *L. plantarum*-pLP503-TTFC moderate titres of TT-specific serum IgG were measured. The *L. casei* and *L. plantarum* strains were sampled efficiently by the M-like cells found in the nasal tracts and induced substantial levels of immunoglobulins in serum. The variations in both the levels of TTFC expression, sustainable levels of non-degraded TTFC and the persistence in the G.I.-tract between the recombinant strains may have pivotal influences on the potency of the recombinant vaccine and may account for the often surprising observations distinguishing the *L. plantarum* and *L. casei* strains.

Discussion

The *L. plantarum* pLP401-TTFC recombinant strains, expressing relatively low levels of surface-exposed TTFC, were immunogenic following i.n. administration, though it was necessary to both prime mice (on days 1-3) and subsequently boost twice (on days 28-30 and 49-51) before antigen-specific responses were measurable. Although surface expression of TTFC would enable direct binding by Igs present on the surface of B cells, potentially augmenting immunogenicity, this surface antigen-

expression may be particularly susceptible to low pH, bile-acid or proteolytic environments encountered by vaccine-vectors following mucosal immunisation. Interestingly however, mice primed as above, with the *L. plantarum* pLP401-TTFC did induce TTFC-specific serum IgG (with more rapid kinetics than responses obtained from naive mice), when boosted as early as days 28-30 using the *L. plantarum* pLP503-TTFC transformant instead. This observation implies that antigen-specific sensitisation of the mice has in fact occurred following administration of *L. plantarum* transformed with pLP401-TTFC, although enhancement, maturation or measurability of the immune response is for numerous reasons prevented from occurring. This would suggest that high levels of "stable" surface expression may not be as critical as accumulated intracellular antigen levels, in order to minimise antigen degradation and ensure sufficient antigen remains available to immune inductive sites.

The present study provides a significant endorsement for *Lactobacillus*-based vaccines by extending the observations of immunogenicity following nasal immunisation to demonstrate for the first time that oral immunisation of C57BL/6 mice with $5x10^9$ *L. plantarum* expressing TTFC intracellularly induces TTFC-specific serum IgG responses. In contrast, BALB/c or C57BL/6 mice immunised orally with the *L. casei* transformants (expressing TTFC either intracellularly or surface-anchored).

L. casei and *L. plantarum* were selected for study due to their different periods of persistence in the G.I. tract. The differences in persistence may explain the differences found in immunogenicity.

It is thought that there may be several factors explaining the *L. plantarum* (cell surface expression) results. Firstly, the antigen may be expressed at lower amounts at the cell surface than intracellularly (almost a difference of an order of magnitude) and so there may be a dosage effect. In addition, there are enzymes present in the lumen that degrade proteins, and so the cell surface proteins are more susceptible to proteolysis. In addition, the protein may have had a slightly different conformation on the cell surface from the equivalent intracellular protein, and furthermore such antigens on the cell surface may change activities at the cell surface, for example with regard to adherence.

The antigen delivery vehicles described emphasise the need to define host-vector combinations and evaluate the impact cell viability, cell numbers, adhesion to the mucosa and the mechanism of triggering of the immune system has on the immunogenicity of the recombinant lactobacilli. This first demonstration of TTFC immunogenicity following delivery of recombinant lactobacilli by the oral route has endorsed safe *Lactobacillus*-based oral neonatal vaccines to combat, for example, the current 400,000 deaths due to tetanus recorded annually.